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M. TARVER
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13281 U.S.PTO

MAIL STOP PROVISIONAL APPLICATION

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c)

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and State or Foreign Country)
Per	**RYDBERG**	**Hagersten, Sweden**

☐ Additional inventors are being named on the _____ separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

INVENTION OF A FLUORESCENT N-R-EDMAN PROCEDURE FOR ANALYSIS OF N-TERMINAL PROTEIN ADDUCTS WITH SPECTROPHOTMETRIC AND/OR MASS SPECTROMETRIC DETECTION

13441 U.S.PTO 60/562985 041904

CORRESPONDENCE ADDRESS

Direct all Correspondence to:

☒ Customer Number 00466

Type Customer No. Here

Firm Name & Address:

Young & Thompson
745 South 23rd Street
Arlington, VA 22202
USA
Telephone: 703-521-2297 - Facsimile: 703-685-0573

ENCLOSED APPLICATION PARTS (check all that apply)

☒	Specification	*Number of Pages:* 10	☐	CD(s), Number	
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☒	Application Data Sheet			**Abstract**	

METHOD OF PAYMENT OF FILING FEES FOR PROVISIONAL APPLICATION FOR PATENT

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Respectfully submitted,

Docket No.: **1523-1010**

By: _____

Date: **April 19, 2004**

Eric Jensen, Reg. No. 37,855
Telephone (703) 521-2297

EJ/yr

PROVISIONAL APPLICATION FILING ONLY

Y&T April 19, 2004

Invention of a Fluorescent N-*R*-Edman procedure for analysis of N-terminal protein adducts with spectrophotmetric and/or mass spectrometric detection

Abstract

We herby introduce a novel method for analysis of electrophilic compounds measured *in vivo* as their corresponding adducts by means of the so-called "Fluorescent N-*R*-Edman procedure". This invention is based on the original observation that N-alkylated N-terminal protein adducts are detached with high selectivity from adducted proteins as their corresponding N-alkyl-valine phenylthiohydantoines after derivatisation with phenyl isothiocyanate (PITC) or pentafluorophenyl isothiocyanate (PFPITC) in the so called N-alkyl Edman procedure. In the Fluorescent N-*R*-Edman procedure fluorescent Edman reagents are used (i.e. the isothiocyanates; FITC ,DNITC and DABITC), detached as their corresponding thiohydantoines which has been isolated from tested N-substituted amino acids model peptides and adducted protein. The analytes were separated on both liquid chromatography (LC) and capillary electrophoresis (CE) and detected by e.g, mass spectrometry (MS) and fluorescence spectroscopic techniques. Low limit of detection (LOD) was obtained using LC-MS/MS techniques (low fmol level on a standard instrument) and a potency for measurements down to low zmol (10^{-21}) levels utilizing CE-laser induced fluorescence (LIF) analysis.

Due to the mild and non-discriminating conditions utilized for the Fluorescens N-*R*-Edman procedure its applicability range is wider than for all earlier existing methods for measurements of N-terminal protein adducts. This new method have excellent sensitivity and specificity, the range for measurement spans from adducts with low molecular masses to adducts that are thermo labile, have high molecular weight and/or have high polarity.

This new method should have the potency for routine analysis for hygienic surveillance, medical purpose and for analysis of forensic substances as their *in vivo* formed electrophillically reactive metabolites.

Background to the invention

It has been demonstrated earlier that *in vivo* electrophilic compounds can be monitored by measuring the products (adducts) of their reaction with proteins, in particular hemoglobin

(Hb) (*1-5*). Important nucleophilic sites in Hb which are reactive under physiological conditions are the imidazole nitrogen atoms in histidine residues, sulfur atoms in cysteine and methionine residues, oxygen atoms in carboxyl groups and in hydroxyl groups in tyrosine and serine residues, and the α-nitrogen atoms in the N-terminal valine residue of all four chains of human Hb (*6*).

The so called N-alkyl Edman procedure was developed for measurements of adducts (mainly low molecular weight adducts) to N-terminal valine residues in Hb (*7*). This method was based on the original Edman degradation procedure (*8,9*) used for protein sequencing . It was observed that N-terminal valine N-alkylated with a radioactively labelled 2-hydroxyethyl moiety from ethylene oxide was released spontaneously as a phenylthiohydantoin (PTH) under the conditions (pH >7) employed for the coupling reaction between phenyl isothiocyanate (PITC) and protein. The released PTH could be separated from unmodified N-terminal valine residues, as well as from the rest of the protein by extraction.

This observation led to the development of the N-alkyl Edman procedure for mass spectrometric (MS) quantitation of Hb adducts (*10*). Because of its usefulness, the N-alkyl Edman method has been applied in a number of laboratories for research purposes, dose monitoring and hygienic surveillance (*11-16*).

A brief description of the N-alkyl Edman procedure is presented in **Figure 1**. A sample of the globin (isolated from red blood cells by acid precipitation) is dissolved in formamide and pentafluorophenyl isothiocyanate (PFPITC) is then added, together with a small amount of aqueous 1 M NaOH in order to obtain a near neutral solution. The mixture is maintained at room temperature overnight, after which the temperature is raised to 45 °C for a couple of hours (*17*). The pentafluorophenylthiohydantoin (PFPTH) derivative of the terminal N-alkylvaline residues are released in high yield by this procedure and subsequently isolated by extraction.

Although the N-alkyl Edman procedure has become an established method for analysis of N-substituted haemoglobin adducts, the method is connected by limitations, e.g., the range of adducts that can be analysed. Small adducts, e.g., ethylene oxide and propylene oxide can be quantified at nmol/g globin level, which is sensitive enough for measurement of so called background adduct levels (levels without known exposure). However, adducts with a few polar groups are more difficult to measure, mainly because of elution problems in the gas chromatographic (GC) system prior to the MS detection. Some of these limitations can be solved, e.g., by further derivatisation (Paulsson et al), this approach is both time consuming and demands introduction of new steps to be developed for each specific adduct. Adducts

with; high molecular weigh (>700 mass units), many polar groups and/or are thermo labile will be extremely difficult to analyse with the GC-MS based N-alkyl Edman procedure.

In order to overcome these problems and to optimize the sensitivity and increase the applicability range the "Fluorescent N-*R*-Edman procedure" has been developed. This invention is based on the principles of the original N-alkyl Edman procedure, alkylated N-terminals can be detached and measured as their corresponding thiohydantoin derivatives after coupling with isothiocyanate Edman reagents.

In contrast to the N-alkyl Edman procedure, this method is based on liquid chromatographic systems, e.g LC and capillary electrophoresis (CE) techniques with MS and/or fluorescence spectroscopic detection methods. Due to the mild and non-discriminating conditions utilized for the Fluorescens N-*R*-Edman procedure its applicability range is much wider compared to the N-alkyl Edman method. The introduction of fluorescent isothiocyanate reagents provides new possibilities for adduct measurements e.g. capillary electrophoresis with laser induced fluorescence detection (CE-LIF, ref) and HPLC with fluorescence detection for N-terminal protein adducts. The use of fluorescent isothiocyanates has been used for peptide sequencing (*18,19*) at nmolar levels by use of e.g., fluorescein isothiocyanate in combination by CE-LIF (*21*), in the study performed by Ireland *et al* the LOD were established to be on the low zmol level.

The Fluorescent N-*R*-Edman procedure is further improved by the utilization of ionisable groups in the applied reagents, e.g., tertiary amines and carboxyl groups, which provides possibilities to enrich the analytes on ion-exchangers, facilitates CE separation and also delivers higher affinity for ionisation in the MS, providing higher sensitivity. The principles of the Fluorescent N-*R*-Edman procedure is presented in **Figure 2.**

In order to compare the Fluorescent N-*R*-Edman procedure with the reagents that are used in the original N-alkyl Edman method and also recently has been applied for LC-MS/MS analysis (Ph.D., Hubert Vesber, using PFPITC, precented in Anaheim, CA May 2004, at the ACS meeting, acrylamide section), a comparative study were performed. The reagents for the N-alkyl Edman procedure, phenyl isothiocyanate (PITC) and pentafluorophenyl isothiocyanate (PFPITC) were evaluated towards three fluorescent isothiocyanate reagents; fluorescein isothiocyanate (FITC), 4-N,N-dimethylaminoazobenzene 4'-isothiocyanate (DABITC) and 4-dimethylamino-1-naphthyl isothiocyanate (DNITC) (se **Figure 3**). The tested reagents were reacted with valine and N-methyl valine, models for non-substituted N-terminal globin and N-substituted N-terminal valine in globine, *N*-(2-carbamoylethyl)-valine were reacted with PFPITC. The formed analytes; phenyl-(valin and N-

methylvaline)thiohydantoin (PTH-Val and PTH-MeVal), pentafluorophenyl-*N*-(2-carbamoylethyl)-valine thiohydantoin (PFPTH-AAVal), fluorescein-(valin and N-methylvaline)thiohydantoin (FTH-Val and FTH-MeVal), 4'-N,N-dimethylaminoazobenzene-4-(valin and N-methylvaline)thiohydantoin (DABTH-Val and DABTH-MeVal) and 4-dimethylamino-1-(valin and N-methylvaline)thiohydantoin (DANTH-Val and DANTH-MeVal) were then compared by measurements at various pH on UV, HPLC and LC-MS/MS. The fluorescent analytes were also measured on fluorescence spectroscopy (excitation and emission spectra) and on CE with diode array detection. As it was observed that one of the selected fluorescent reagent were superior in comparison with the rest of the reagents, this reagent were also evaluated on alkylated model peptides and globine adducted with acrylamide (AA), glycidamide (GA), propylene oxid (PE), cholesterol-5α,6α-epoxide (Chol-EO) and 2-octadecyl-oxirane.

Experimental

Material and Methods

Chemicals. Structures of compounds **1-14** are given in **Figure 4**.Flureseeine isothiocyanate (isomer I, <90%), pentafluorophenyl isothiocyanate (PFPITC) and phenyl isothiocyanate (PITC, purum) were obtained from Fluka. 4-N,N-Dimethylaminoazobenzene 4'-isothiocyanate (DABITC) and 4-dimethylamino-1-naphthyl isothiocyanate (DNITC) were obtained from Acros. Cholesterole-5α,6α-epoxide, octadecyl-epoxide, L-Valine (Val), L-valinamide ($ValNH_2$), *N*-methyl-*D,L*-valine (MeVal) and 5-isopropyl-3-phenyl-2-thiohydantoin (Val-PTH, **1**) were obtained from Sigma. *N*-Methylvalylleucylanilide (MeValLeu-NHφ > 99 %) and valylleucylserine [ValLeuSer (H-Val-Leu-Ser-OH) 95%) were obtained from Bachem (Bubendorf, Switzerland). ($^2H_3$)Acetonitrile (99.8 % ^2H), (^2H)chloroform (99.8 % ^2H), deuterium oxide (99.9 % ^2H, ^2H_2O), and ($^2H_4$)methanol (99.8 % ^2H) were obtained from CIL (Andover, MA). 5-Isopropyl-1-methyl-3-phenyl-2-thiohydantoin (MeVal-PTH, **6**), 5-isopropyl-3-pentafluorophenyl-2-thiohydantoin (Val-PFPTH, **12**) and 5-isopropyl-1-methyl-3-pentafluorophenyl-2-thiohydantoin (MeVal-PFPTH, **13**) were synthesised as described earlier (*20*). Glycidamide (GA) were synthesised. All other chemicals and solvents were of analytical grade.

Instrumentation, methods for analysis and characterization. ^1H and ^{13}C NMR spectra were recorded on a JEOL GSX 270 instrument at 270 MHz. All solvents used were fully deuterated, TMS was added as internal standard in chloroform, acetonitrile and methanol.

Methods and instrumentation for LC-MS/MS analysisis. The LC-MS system comprized a Rheos 4000LC pump (Flux Instruments, Basel, Switzerland) interfaced with a LCQ (ThermoQuest, CA, USA). The MS was operated in electrospray ionisation (ESI) mode. The mobile phase consisted of 1:1, H_2O:acetonitrile at an isocratic flow at 200 µL/min. The ion-source temperature was 120 °C, capillary voltage 3.5 kV and the cone voltage varied between 25 and 140 V. Nitrogen was used as drying gas at a flowrate of 250 L/h. Both the positive- and the negative-ion mode were used. MS-MS was performed by utilizing collision-induced dissociation (CID) of the [M+1] ion.

Studies of retention times of compounds 4-10 were carried out on HPLC, on a Shimadzu LC-4A connected with a Kromasil LC-18 column (250 x 10 mm) and a Shimadzu SPD-2AS detector (λ = 268 nm, D_2 lamp). Flow rate = 2.5 mL/min, loop 0.7 ml eluted with 2 % aqueous acetonitrile buffered with 0.02 % TFA.

TLC was performed using silica gel 60 f-254 plates (SiO_2, Merck), spots were developed with UV and at long wave (378 nm) Melting points were determined on a Büchi 535 instrument. Mikrokemi AB, Uppsala, Sweden, performed elementary analyses. Measurements of pH were carried out on an Orion EA 920 pH-meter equipped with a Ross 8130 glass electrode.

The CE separation was performed on an HP 3D CE with a five channel diode array UV detector (Agilent, CA, USA). A fused silica capillary (i.d. 50 µm, o.d. 375 µm) with a total length of 64 cm and an effective length of 56 cm was used. The separation voltage was +30 kV, resulting in an separation current of 32 µA. The buffer system consisted of a 17 mM phosphate buffer (adjusted to pH 7) containing 20 mM SDS.

*Synthesis of N-Me(4-Me$_2$N-Naftyl)valin-tiohydantoin (*5*,* DMAP-MeVal *).* D,L-MeVal (prepared according to Rydberg *et al* 93, 93.0 mg, 0,708 mmol) was alkalized with KOH (0,4 mmol) and dissolved in 0,5 M $KHCO_3$ (3 ml) and dioxan (2 ml). The solution was heated to 45 °C with magnetic stirring and p-dimethylnafthylisothiocyanate (DNITC, 91.3 mg, 0.40 mmol) dissolved in dioxane (2 ml) was added and the solution. The reaction was followed on TLC (SiO_2, toluene and 2:1 toluene / ethyl acetate) with a blank reference (DNITC dissolved in the solvent mixture). After 60 min the reaction was completed, no DMNaf-ITC could be detected on TLC and the formed product gave fluorecense at long wave under the UV-lamp. The reaction was extracted with toluene and purified by column chromathography ((SiO_2, toluene and 2:1 toluene / ethyl acetate), to yield 130 mg, 96 % yield. The product was recrystallized from ethanol/water (1:1), to yield white crystals (100 mg, 73 % yield), for NMR, see **Figure 5.**

*Synthesis of 4-Me_2N-Naftyl)valin-tiohydantoin (***4**, DMAP-Val). L-Valine (Sigma, 804 mg, 6.86 mmol) was alkalized with KOH (3.43 mmol) and dissolved in 0,5 M $KHCO_3$ (3 ml) and dioxan (3 ml). The solution was heated to 45 °C with magnetic stirring and DMNaf-ITC (88 mg, 0.385 mmol) dissolved in dioxane (2 ml) was added to the suspended solution. The reaction was followed on TLC (SiO_2, Ethyl acetate / MeOH 4:1, toluene and 2:1 toluene / ethyl acetate) with a reference whithout addition of Valine. After 60 min the reaction was in principal completed, no DMNaf-ITC could be detected on TLC and the formed product, the 4-Me_2N-Naftyl)-tiocarbamoyl-valinate gave fluorecense at long wave under the UV-lamp (tailing spot on TLC eluted with Ethyl acetate / MeOH 4:1). The reaction was acidified with hydrochloric acid (3 ml conc.) and the cyclisation was interrupted after 2 h, at 45 ° by neutralization with solid $KHCO_3$ until no more carbodioxide was formed. The reaction mixture was diluted with water (100 ml) and the product was purified by extraction with $CHCl_3$ (125 ml). The organic phase was dried with Na_2SO_4, filtered and evaporated to yield 126 mg, yield 0.96 % as a white solid.

The syntheseis of compound **6-14** were performed as described above, the products were confirmed by ^1H and ^{13}C NMR spectra and by LC-MS/MS (ESI) positive and negative ionization, eg., **Figure 6,** ^{13}C NMR spectra of FTH-MeVal, **Figure 7,** ^1H NMR spectra of FTH-MeVal and **Figure 8,** ^{13}C NMR spectra of FTH –MeVal.

Results and discussion

In order to evaluate the potency of the tested isothiocyanates the relative response of compound **1-14** (see **Figure 3**) were measured and compared using fluorescence spectroscopy (compound **4-10**), HPLC, CE and UV spectroscopy. For the main part of these studies pH were alternated below and above the pKa for the respective analyte (for pKa values see **Figure 4**). The studies on relative fluorescence were performed on the fluorescent analytes (compound **4-10** in **Figure 4**) by measurements of excitation and emission spectra. The results from this study is presented in **figure 9-12**, FTH-Val and FTH-MeVal gave both in principle identically and high response at pH above 5, showing that the adduct, e.g., the methyl in FPT-MeVal (**9**) does not effect the spectroscopic properties in comparison with FTH-Val (**8**). In these studies FTH-Val and FTH-Me Val gave superior response compared to the other used reagents.

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In order to compare the relative sensitivity on LC-MS/MS compound **1-9** were compared by injecting 5 µl directly in the electrospray interface of the TSQ (n=3 for each compound), the cone voltage were adjusted in order to obtain maximum sensitivity for the each specific analyte. The LC-MS/MS(ESI) measurements were performed using well adopted buffer systems, e.g., 0.1 % TFA, 0.1 % ammonium acetate and 0.3 mM aqueous ammonia. For LOD measurements the flow rate were 200 µl/min of the aqueous buffer/acetonitrile [1:1 (v/v)] the analytes were dissolved in the same buffers and solvent mixtures at concentrations ranging from 10 µg/ml down to 1 ng/ml depending on their response for each used condition. The results from this study are summarized in **Figures 14-20** and in **Table 1**. As compound **8** and **9**, FTH-Val and FTH-MeVal, gave superior response under the tested conditions for the LC-MS analysis in combination with excellent column separation on HPLC (C_{18}-colums, especially when eluted at acidic conditions), they were selected for further studies. In order to investigate the applicability range for this reagent, valine and globine were adducted with glycidamide, propylene oxide and the high molecular weight adducts cholesterole-5α,6α-epoxide and octadecyl-epoxide. The formed thiohydantoines (compounds **10-14**) were characterized on LC-MS/MS and the adducted globins could be measured after derivatisation (90 min in 0.5 M aqueous $KHCO_3$/ 2-propanol [2/1 (v/v)], followed by size discriminating ultra filtration (MWCO 5000), evaporation filtrate or concentration of the analytes from the filtrate by use of anion exchangers. This approach is not only time saving but is also non-discriminating especially for analysis of adducts with high polarity as they often are lost or gives poor yields on liquid/liquid or solid phase extraction clean up steps.

In order to evaluate the possibility to measure N-terminal adducts with such sensitive techniques as CE-LIF, the separation between F-MeValTH and F-ValTH on CE were evaluated. A complete baseline separation was obtained using the stated conditions e.g., in 17 mM phosphare buffer (adjusted to pH 7) containing 20 mM SDS (see **Figure 21**). The intensity of the fluorescence reaches an optimum around this pH for both FTH-Val and FTH-MeVal, which opens up the possibility to utilize

CE-LIF for adducts measurements down to the low zmol (10^{-21}) levels, which has been shown by Ireland *et. al.* (*21*). In their study 18 of 20 coded FTH-amino acids were separated and analysed with LOD of around 10 zmol. Their results are truly encouraging for miniaturizing the fluorescent-N-*R* Edman procedure, aiming to measure adduct spectra from a few µl blood, easily available by a prick in the fingertip.

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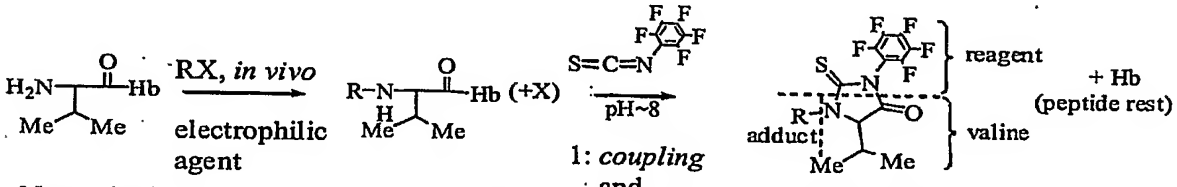
1. Tables

Table 1. Comparison of relative sensitivities obtained by measurements of limit of detection detection LOD on LC-MS/MS(ESI), using various pH modifiers/buffers. LOD were meaured on positive and negative ions with direct injections (5 ul loop). The lowest obtained sensitivity is set to one, and the others are set relative to FTH-MeVal in 0.3 mM NH_3, negative ions measured (marked yellow), this where calculated to be 2,6 fmol.

substance	Positive 0,1% NH4oAc	Positive 0,1% TFA	Positive 0,3mM NH3	Positive Without buffer	Negative 0,1% NH4oAc	negative 0,3mM NH3	negative Without buffer
FTH-MeVal	15,8	3,9	4,6	447,6	13,8	1,0	50,1
FTH-Val	3,6	1,1	n.d	393,1	1,6	n.d	57,3
DABTH-MeVal	60,4	26,9	9,8	377,7	45,1	55,9	386,7
PTH-MeVal	14439,5	7821,4	594,1	36098,9	6618,1	902,5	895,3
NTH-MeVal	940,8	90,9	460,7	932,1	216593,1	15752,2	984,8

Figures:

Figure 1. Principles of the N-alkyl Edman procedure.



N-terminal valine in Hb

N-substituted terminal valine

1: *coupling* and
2: *cleavage*

N-(substituted)Val-PFPTH formed directly if R=alkyl, aryl. Analysis by GC-MS/MS

Figure 2. Principles of the Fluorescent N-*R*-Edman procedure.

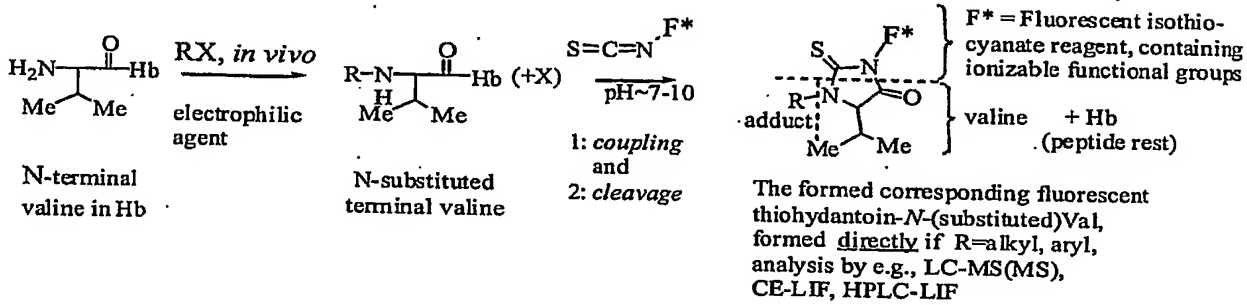
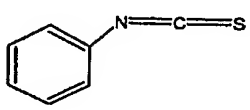
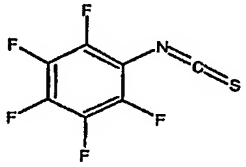


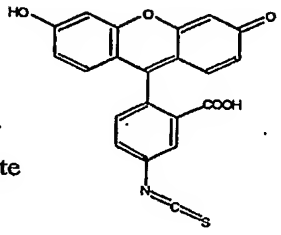
Figure 3. Used isothiocyanate reagents in this study



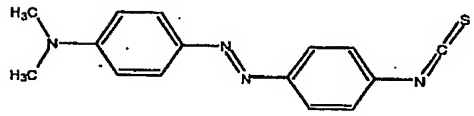
Pheny isothiocyanate (PITC) (Used in the N-alkyl Edman procedure)



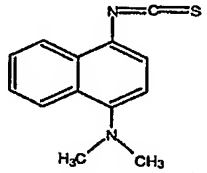
Pentafluorophenyl isothiocyanate (PFPITC) (Used in the N-alkyl Edman procedure)



Fluorescein isothiocyanate (FITC)

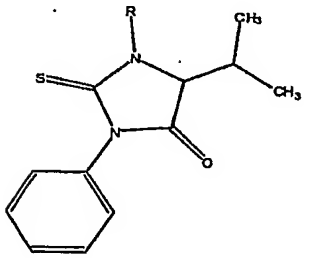


4-N,N-Dimethylaminoazobenzene 4′-isothiocyanate (DABITC)

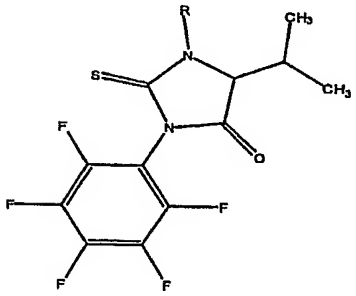


4-N,N-dimethylamino-1-naphthyl isothiocyanate (DNITC)

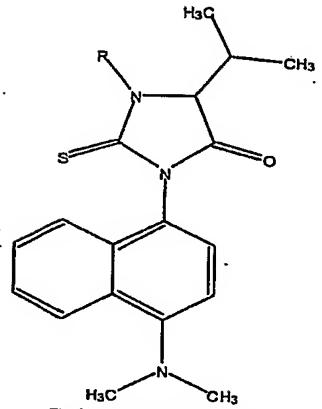
Figure 4. Structures, abbreviations and molecular weights of the studied compounds



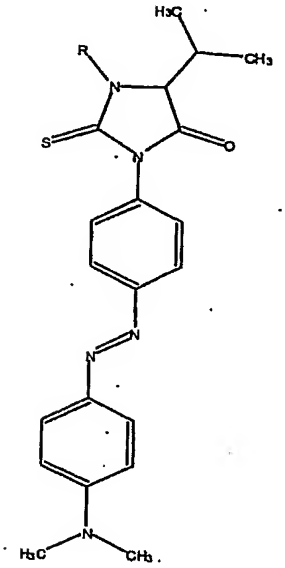
Substance **1**, R=H, PTH-Val, M=234.31 g/mol
Substance **2**, R=Me, PTH-MeVal, M=248.35 g/mol
(non-fluorescent analytes)



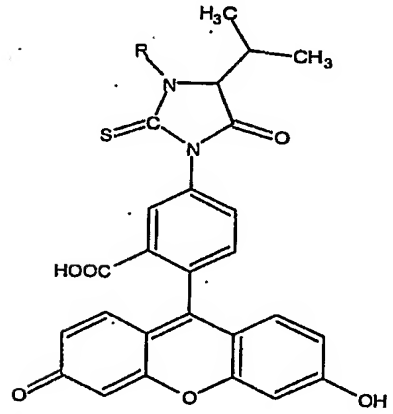
Substance **3**, R= $CH_2CH_2CONH_2$, PTH-AAVal, M=395.35 g/mol
(non-fluorescent analyte)



Substance **4**, R=H, DMAP-Val, M=327.44
Substance **5**, R=Me, DMAP-MeVal M=341.47
(fluorescent analytes, pKa for the dimethylamine amine calculated to be 6, (±0.3)



Substance **6**, R=H, DABTH-Val M=381.50
Substance **7**, **R**=Me DABTH-MeVal M=395.52
(fluorescent analytes, pKa for the dimethylamine amine calculated to be 3.4, (±0.3)



Substance **8**, R=H FTH-Val M=488.51
Substance **9**, R=Me FTH-MeVal M=502.54
Substance **10**, R=$CH_2CH_2CONH_2$ FTH-AAVal M=559.59
Substance **11**, R=$CH_2CH(OH)CONH_2$ FTH-GAVal M=575.59
Substance **12**, R=$CH_2CH[O]CH_2$ FTH-POVal M=546.59
Substance **13**, R=CH2CH[O]CH[$CH_{2\ (n=15)}$]CH_3, FTH-C_{18}EO M=756.99
Substance **14**, R=5a or 6a adduct to cholesterol epoxide: FTH-Chol-O M=891.17
(fluorescent analytes, pKa for the carboxyl group calculated to be 3.27(±0.3)

Figure 5, ^{13}C NMR spectra of DMAP-MeVal

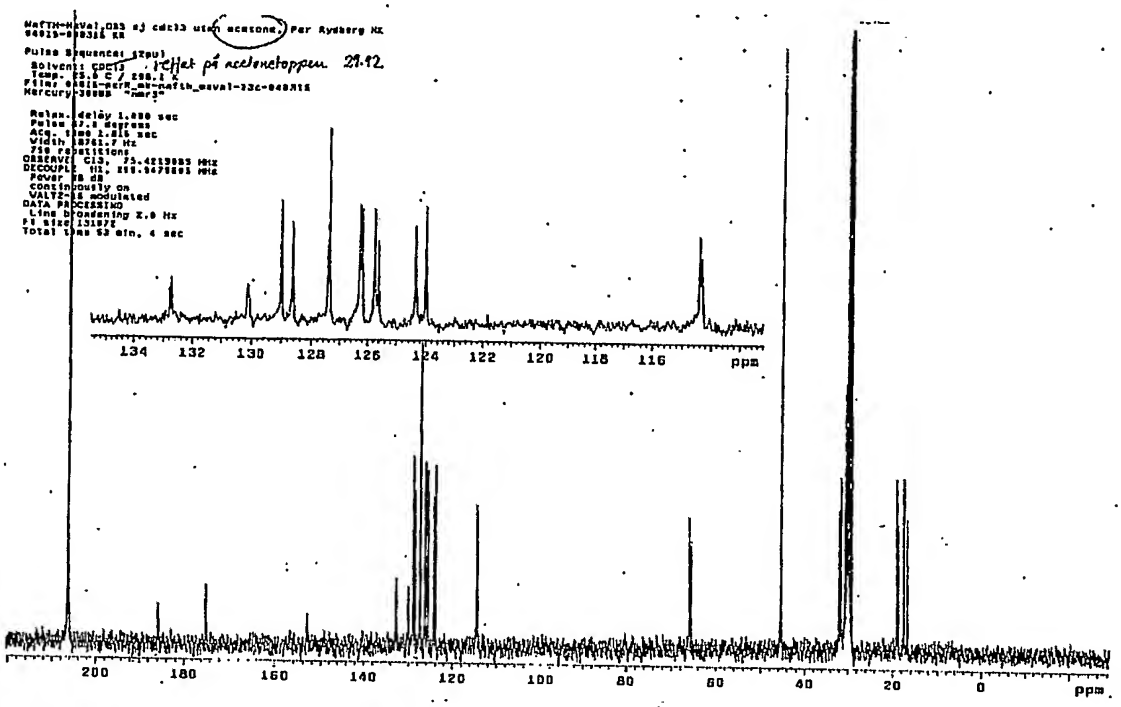


Figure 6, ^{13}C NMR spectra of FTH-MeVal

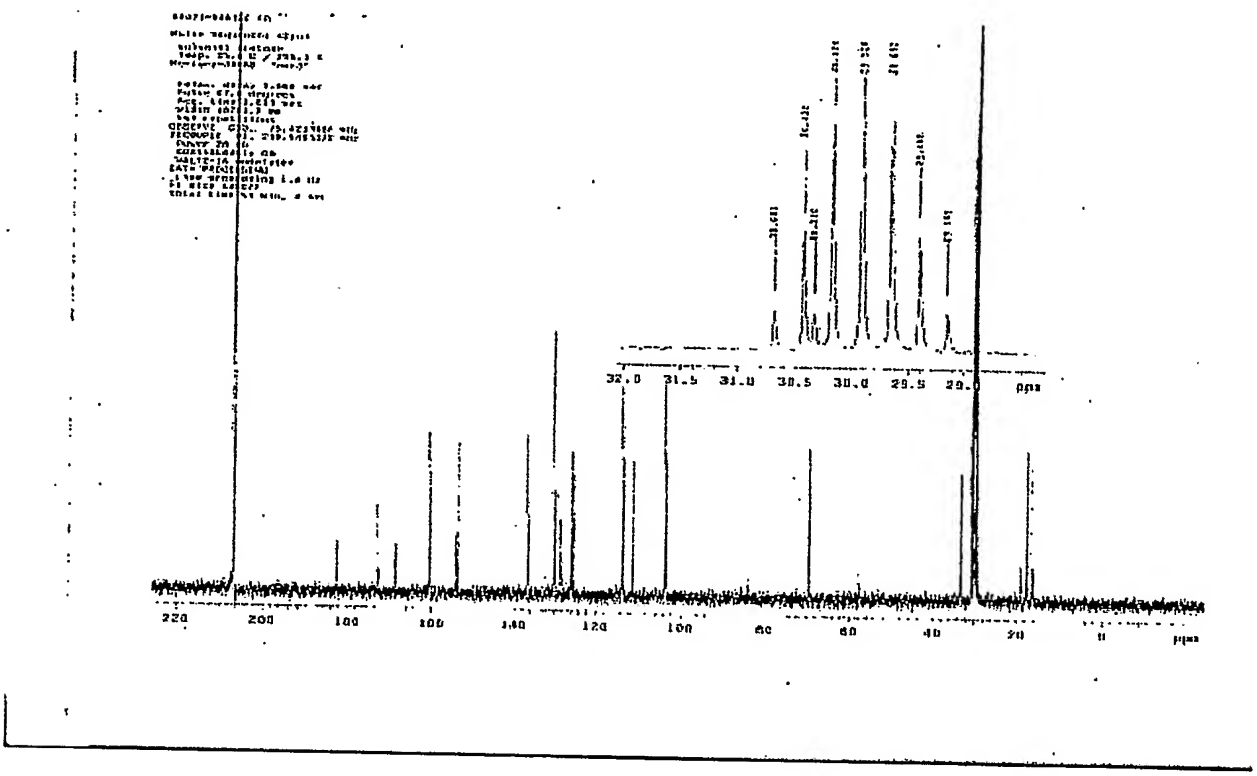


Figure 7, ^{1}H NMR spectra of FTH-MeVal

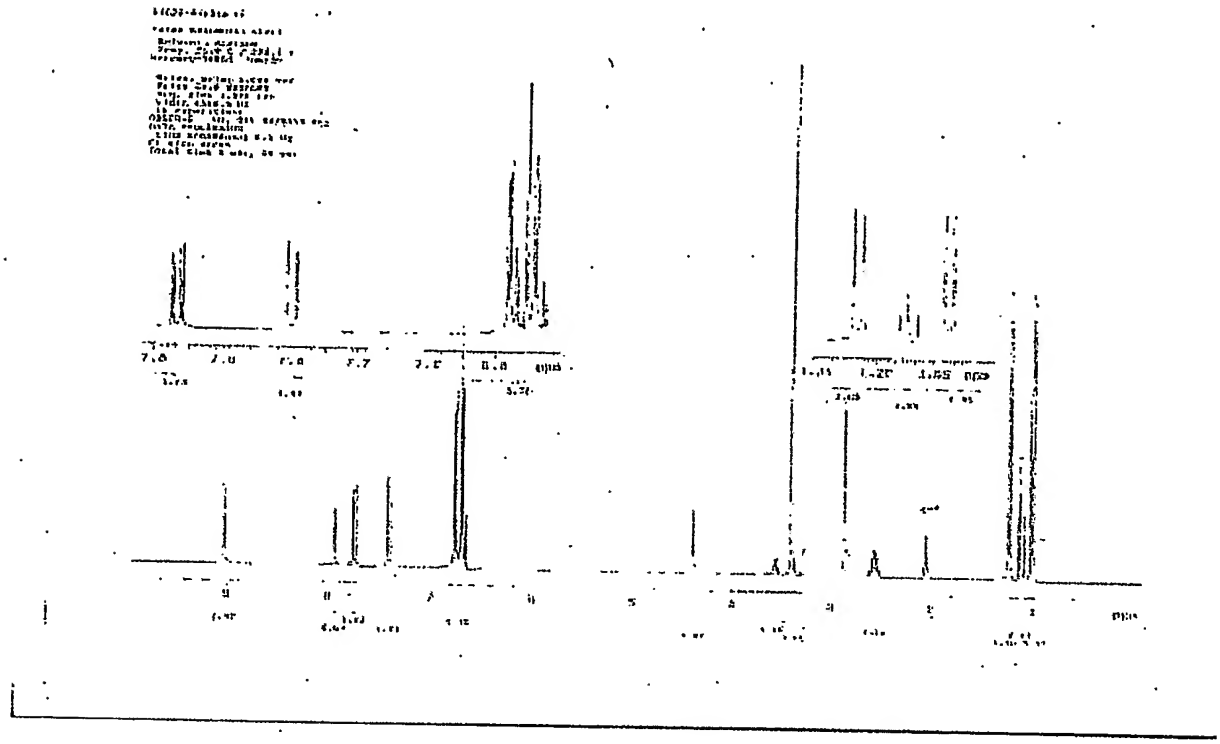


Figure 8, ^{13}C NMR spectra of FTH –MeVal

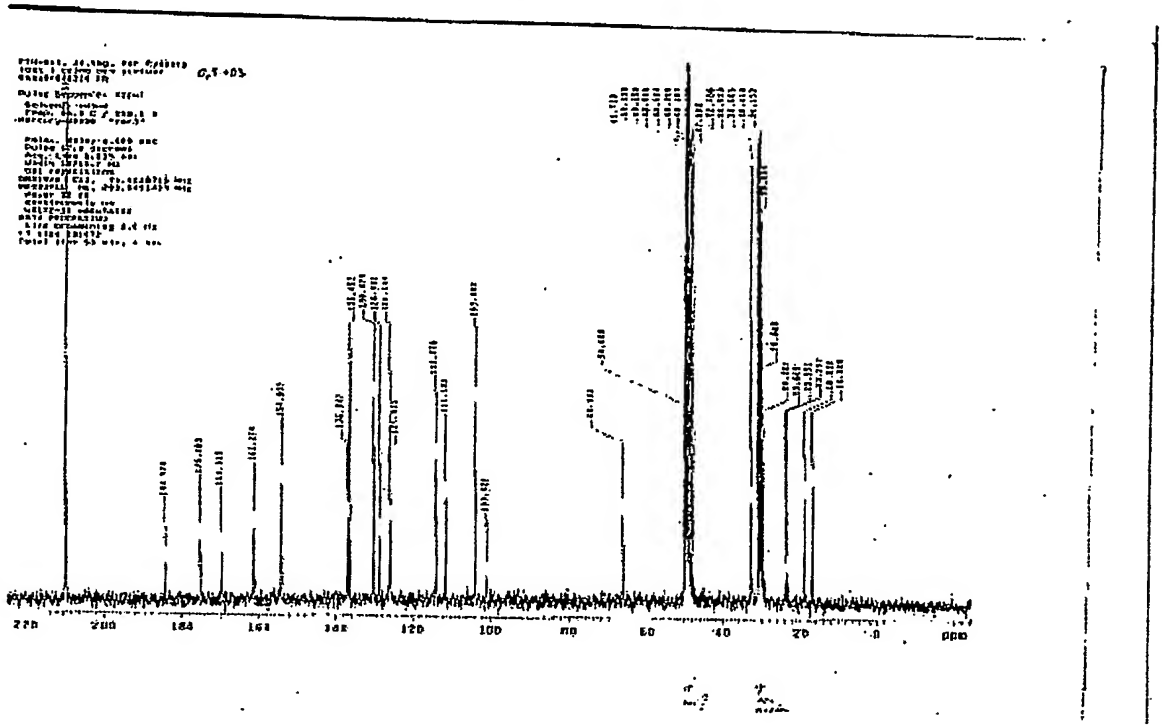


Figure 9. Fluorescens measurements; the excitation and emission spectra of FTH-Val in dry acetonitrile at various concentrations.

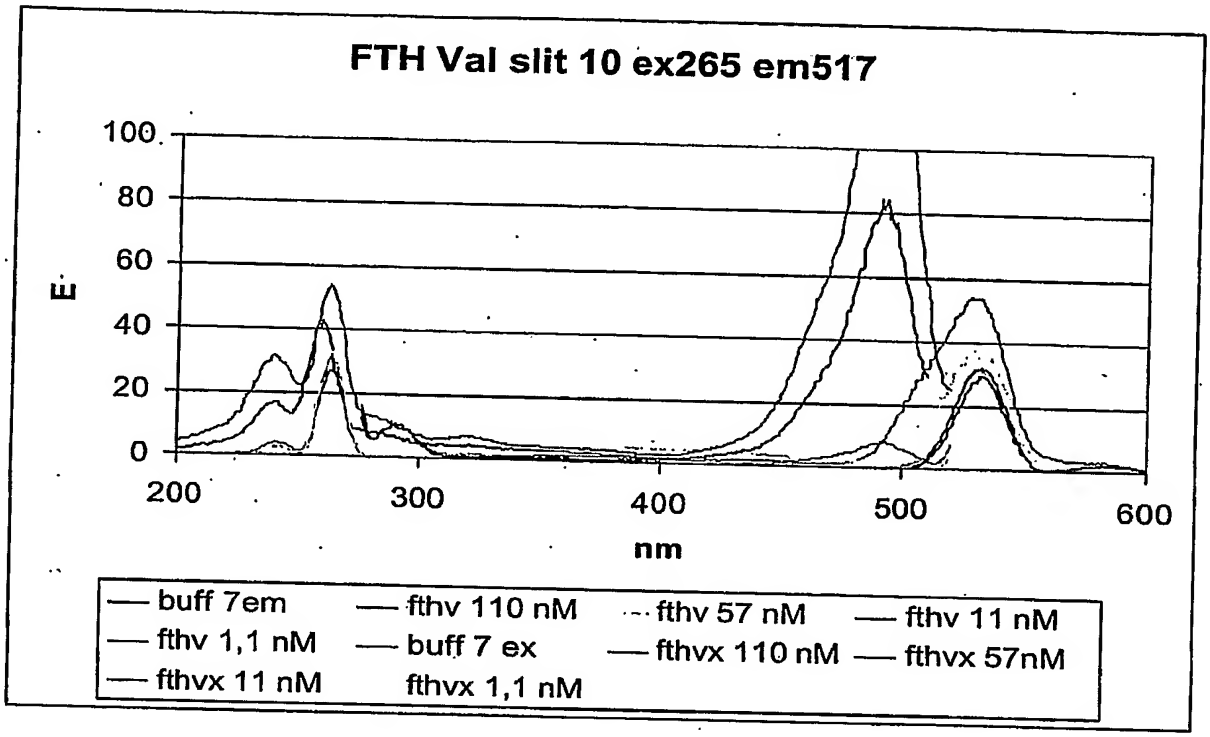


Figure 10. Fluorescens measurements; the excitation and emission spectra of FTH-MeVal in at pH 1, 4 7 and 9 at 0.5 µg/ml.

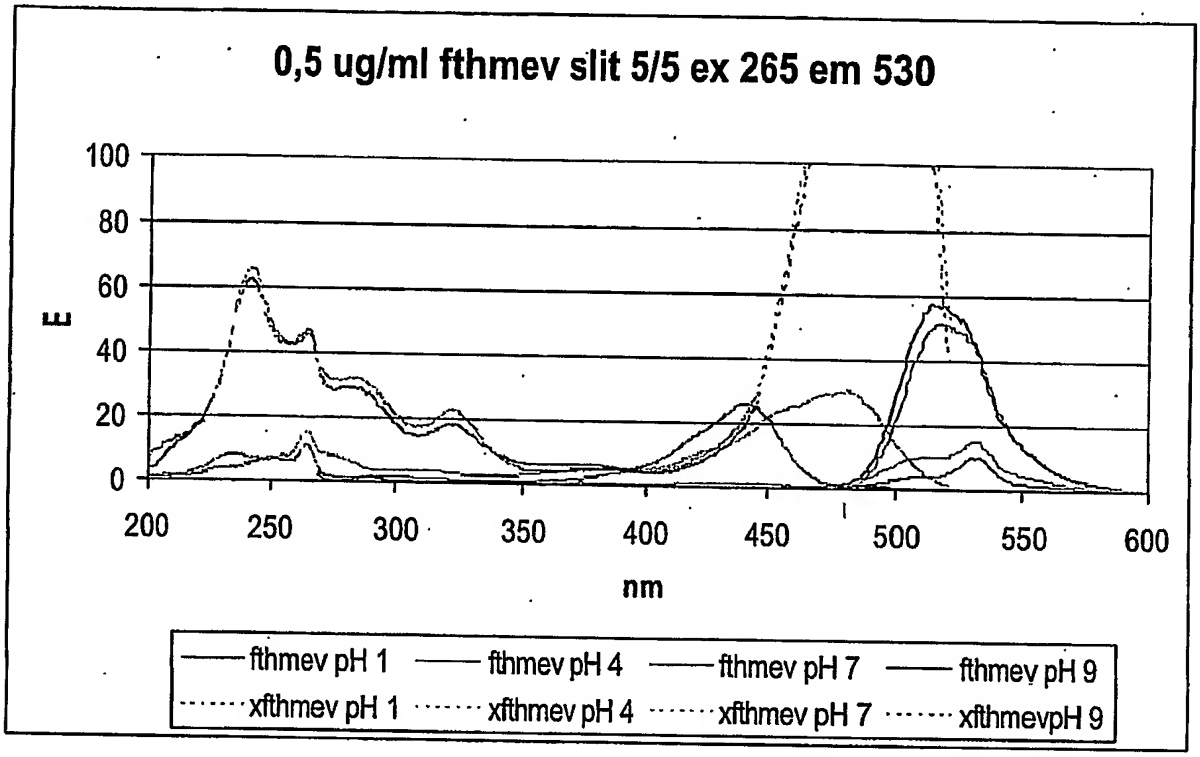


Figure 11. Fluorescens measurements; the excitation and emission spectra of DABTH-MeVal in at pH 1, 4 7 and 9 at 1 µg/ml.

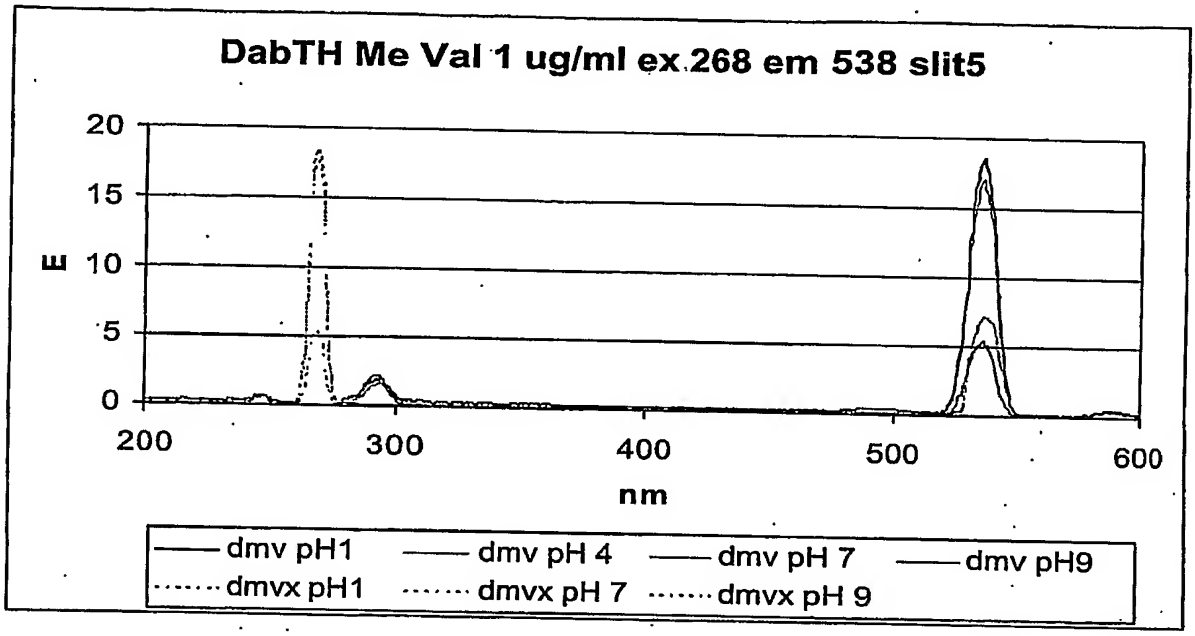


Figure 12. Comparative studies of the excitation and emission spectra of the fluorescent analytes FTH-MeVal, FTH-Val, DABTH-MeVal. DABTH-Val, DNTH-MeVal, DNTH-Val and the reference fluorantene at the given concentrations in dry acetonitrile

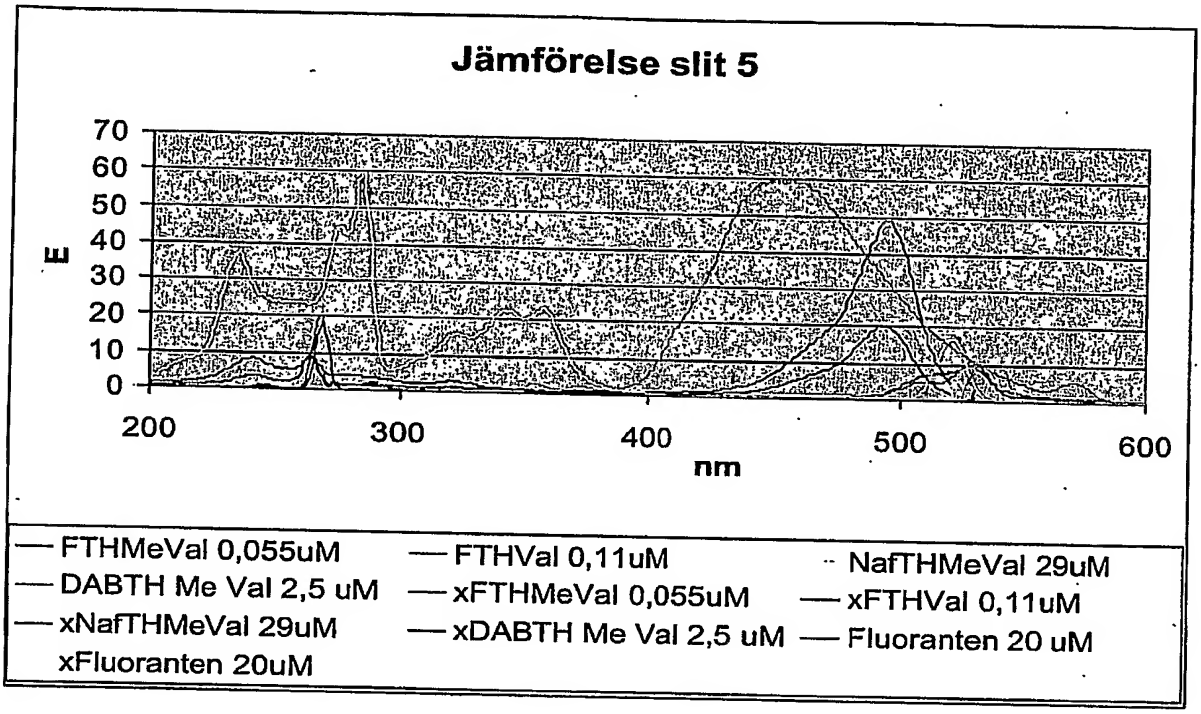


Figure 13. Fluorescens measurements; the excitation and emission spectra of DAMTH-Val at pH 1, 4 7 and 9 at 1 µg/ml.

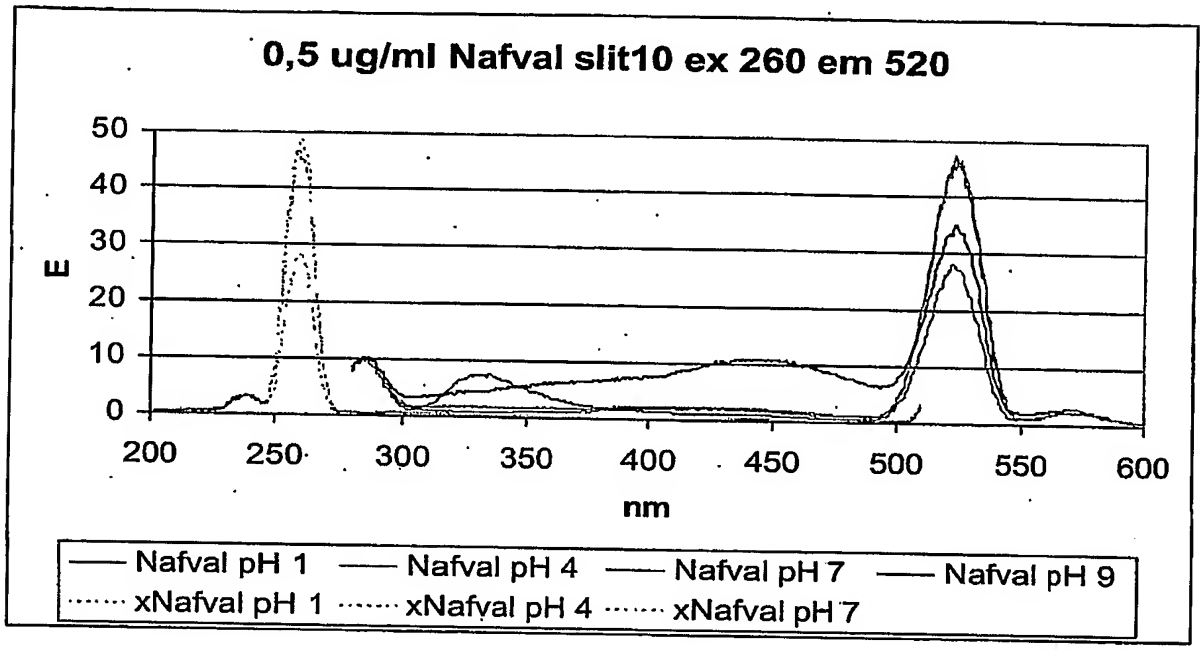
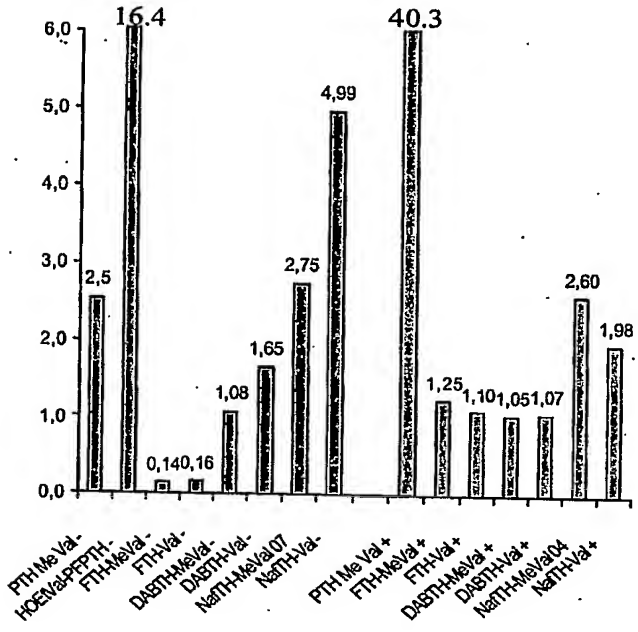


Figure 14. Comparison between the tested analytes by measurements of limit of detection LOD on LC-MS/MS with electrospray ionisation and a 5 ul loop, without buffers, direct injections.



Substans	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision energi (rel. %)
PTH Me Val -	247.0	232.0	40
HOEtVal-PFPTH	367.1	347.1	25
FTH-MeVal -	501.2	457.3	35
FTH-Val -	487.2	443.3	30
DABTH-MeVal -	487.2	379.3	40
DABTH-Val -	380.2	264.3	35
NafTH-MeVal -	340.2	325.2	35
NafTH-Val -	326.1	210.2	35
PTH Me Val +	249.2	176.0	35
FTH-MeVal +	503.3	460.1	40
FTH-Val +	489.2	390.3	40
DABTH-MeVal +	396.2	219.0	40
DABTH-Val +	382.2	205.0	40
NafTH-MeVal +	342.2	327.1	35
NafTH-Val +	328.2	219.0	40

Figure 15. Comparison between the tested analytes by measurements of limit of detection LOD on LC-MS/MS with electrospray ionisation and a 5 ul loop, with an addition of 0.3 mM ammonia (pH around 9) with direct injection.

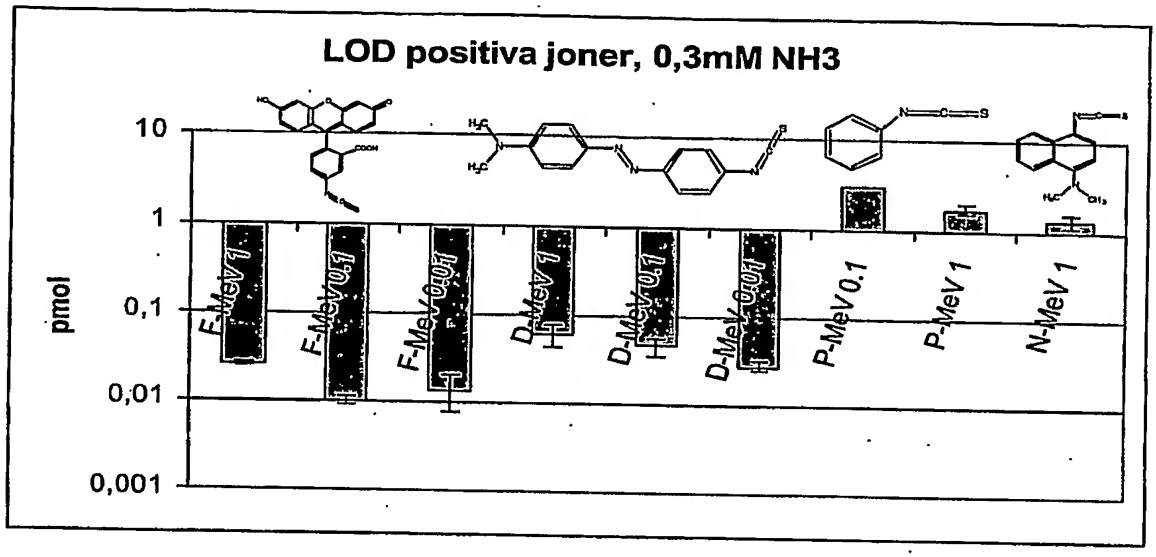


Figure 16. Comparison between the tested analytes by measurements of limit of detection LOD on LC-MS/MS with electrospray ionisation and a 5 ul loop, with an addition of 0.1 % TFA (pH around 1) with direct injection.

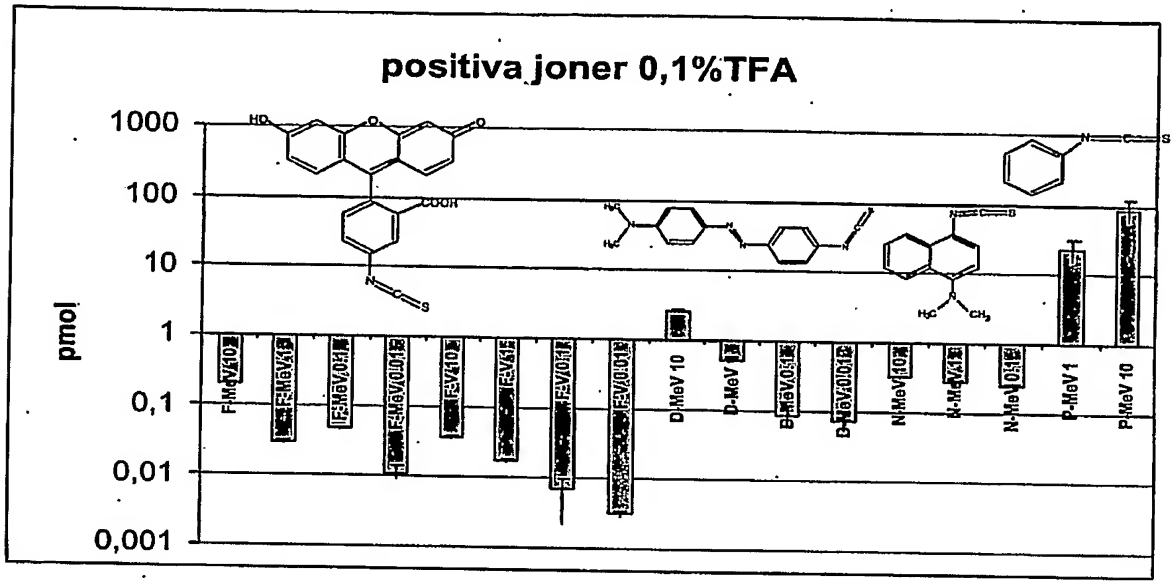


Figure 17. Comparison between the tested analytes by measurements of limit of detection LOD on LC-MS/MS with electrospray and a 5 ul loop, with additions of specified buffers, measured on positive ions with direct injections.

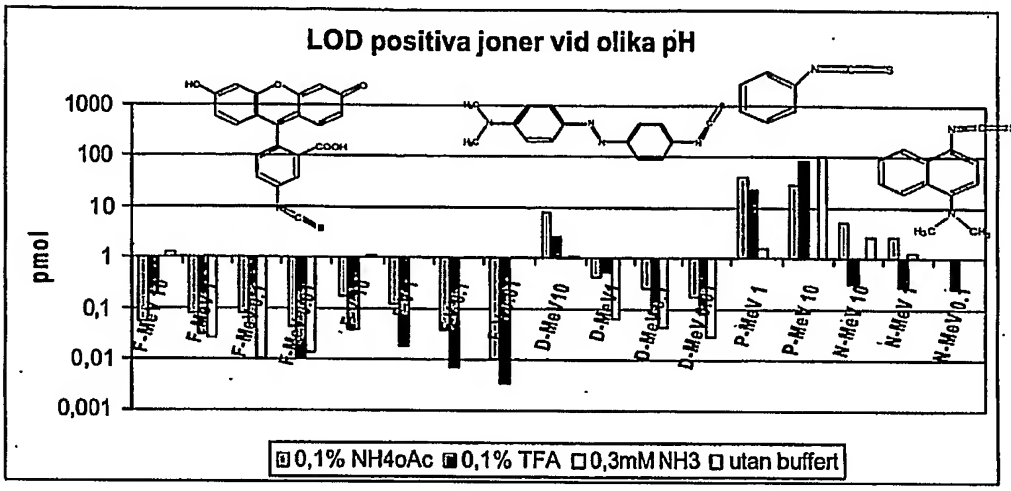


Figure 18. Comparison between the tested analytes by measurements of limit of detection LOD on LC-MS/MS with electrospray and a 5 ul loop, with additions of 0.2 % ammonium acetate (pH 6.96) , measured on negative ions with direct injections.

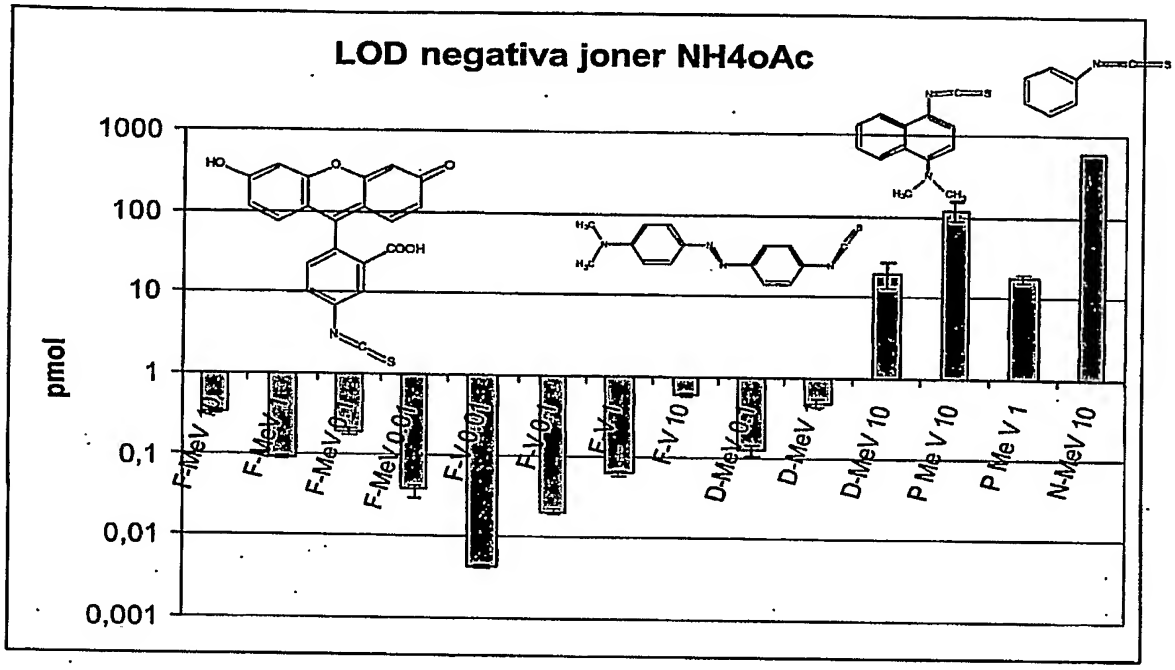


Figure 19. Comparison between the tested analytes by measurements of the limit of detection LOD on LC-MS/MS with electrospray and a 5 ul loop, with additions of 0.3 % ammonia (pH around 9) , measured on negative ions with direct injections.

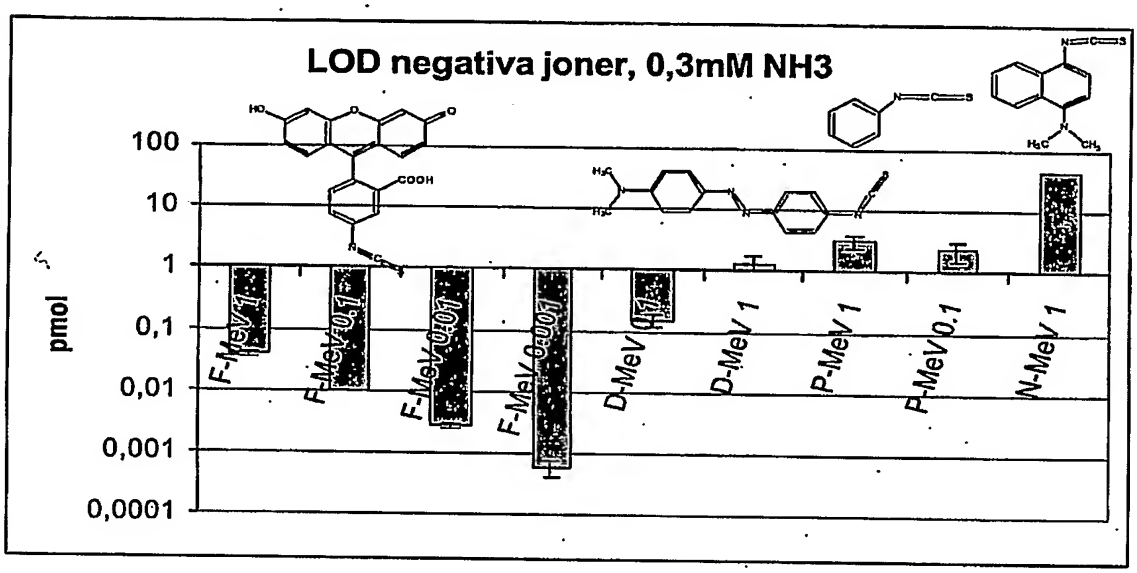


Figure 20. Comparison between the tested analytes by measurements of the limit of detection LOD on LC-MS/MS with electrospray and a 5 ul loop, with additions of various pH modifying buffers , measured on negative ions with direct injections

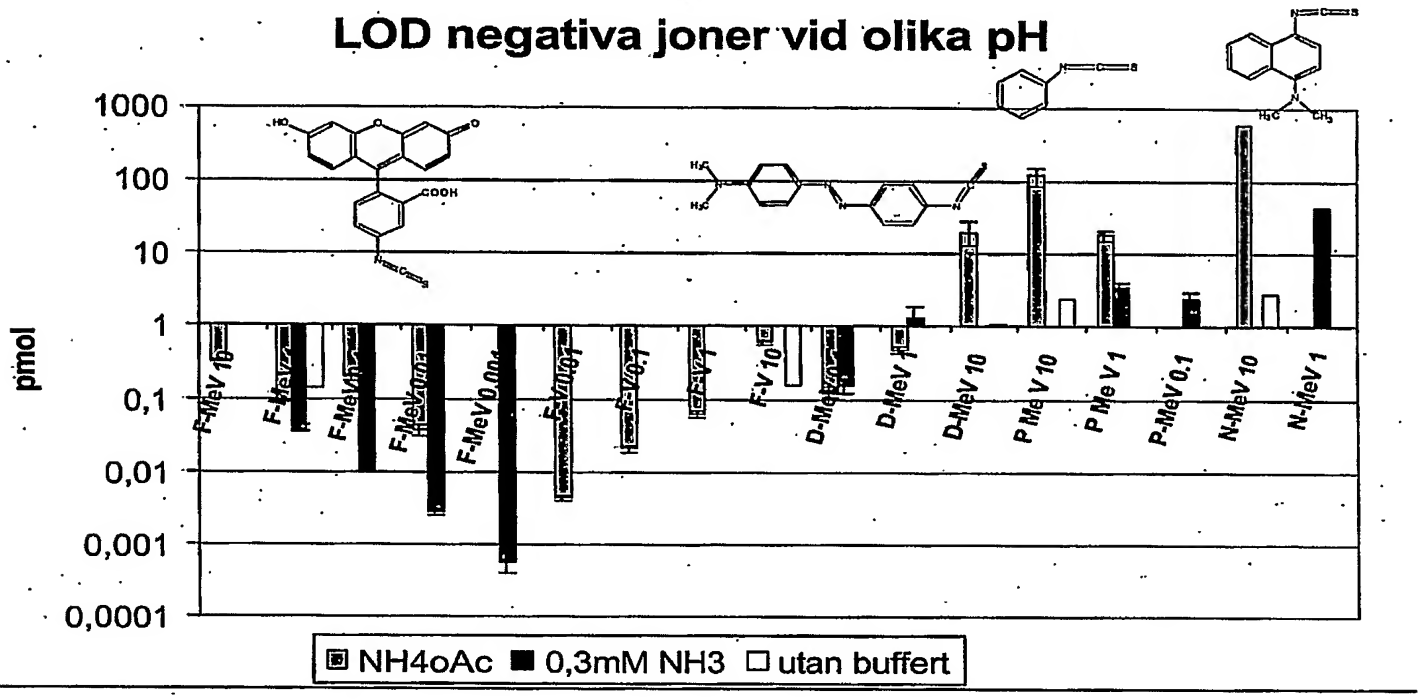
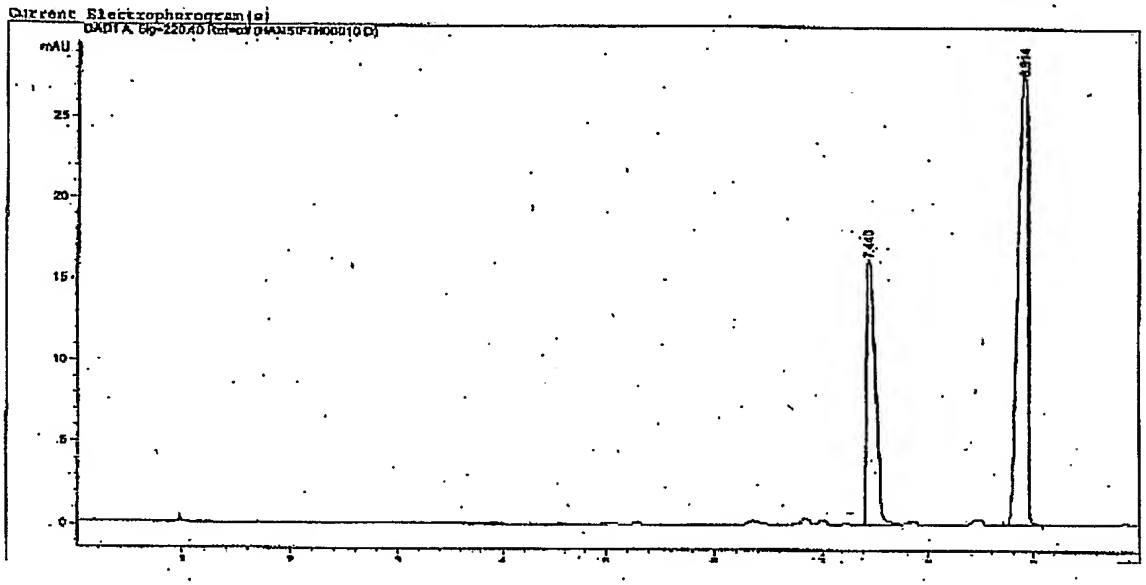


Figure 21. Separation between FTH-MeVal and FTH-Val on Capillary electrophoresis measured with diode array detection at 220 nm.

FTH-Val elutes at 7.44 min and FTH MeVal elutes at 8.91 min (phosphate buffer 17 mM, pH 7, 20 mM SDS). 30 kV, 52 cm capillary, 1 nl injected.



Application Data Sheet

Application Information

Application Type::	Provisional
Subject Matter::	Utility
Suggested Classification::	
Suggested Group Art Unit::	
CD-ROM or CD-R?::	None
Number of CD disks::	
Number of Copies of CDs::	
Sequence Submission?::	None
Computer Readable Form (CRF)::	No
Number of copies of CRF::	0
Title::	INVENTION OF A FLUORESCENT N-R-EDMAN PROCEDURE FOR ANALYSIS OF N-TERMINAL PROTEIN ADDUCTS WITH SPECTROPHOTMETRIC AND/OR MASS SPECTROMETRIC DETECTION
Attorney Docket Number::	1523-1010
Request for Early Publication?::	No
Request for Non-Publication?::	No
Suggested Drawing Figure::	
Total Drawing Sheets::	23
Small Entity?::	Yes
Latin Name::	
Variety Denomination Name::	
Petition Included?::	No
Petition Type::	
Licensed US Gov't Agency::	
Contract or Grant Numbers::	
Secrecy Order in Parent Appl.?::	No

Applicant Information

Applicant Authority Type:: Inventor
Primary Citizenship Country:: SWEDEN
Status:: Full Capacity
Given Name:: PER
Middle Name::
Family Name:: RYDBERG
Name Suffix::
City of Residence:: HAGERSTEN
State or Province of Residence::
Country of Residence:: SWEDEN
Street of Mailing Address:: TOMTRATTSVAGEN 37
City of Mailing Address:: HAGERSTEN
State or Province of Mailing Address::
Country of Mailing Address:: SWEDEN
Postal or Zip Code of Mailing Address:: SE-129 31

Correspondence Information

Correspondence Customer Number:: 000466

Representative Information

Representative Customer Number::	000466

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::

Initial 4/19/04

Foreign Priority Information

Country::	Application Number::	Filing Date::	Priority Claimed::

Assignment Information

Assignee Name::

Street of Mailing Address::

City of Mailing Address::

State or Province of Mailing Address::

Country of Mailing Address::

Postal or Zip Code of Mailing Address::